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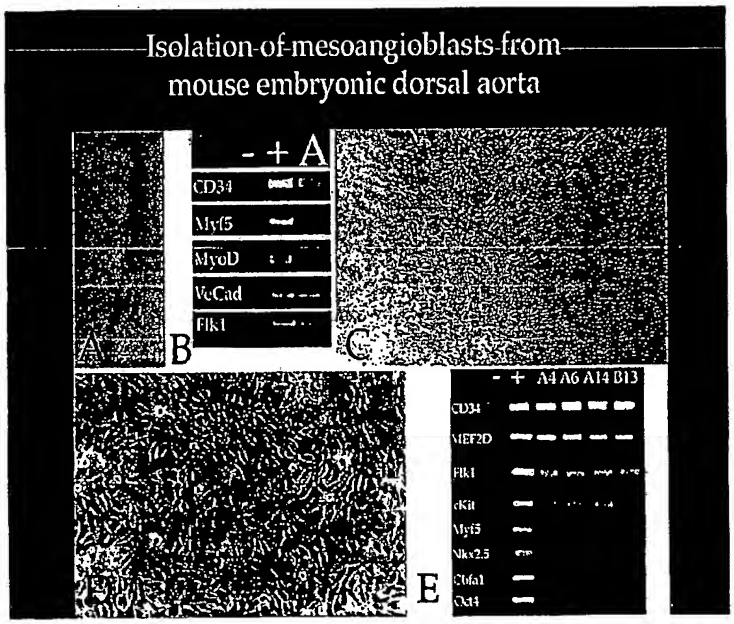

(57) Abstract: Disclosed are novel multipotent stem cells, a method for their isolation and in vitro expansion, processes for their in vitro differentiation and their use for regenerating or repairing biological tissues and as therapeutic agents.

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METHOD FOR ESTABLISHING AND EXPANDING MULTIPOTENT STEM CELLS

The present invention provides a method for establishing and expanding multipotent stem cells ("mesoangioblasts"), which utilizes a combination of cell culture and clonal selection.

The multipotent, self-renewing stem cells according to the invention can be isolated from embryonic, fetal or adult blood vessels, postnatal cord blood and from perinatal or adult bone marrow. Under appropriate conditions the mesoangioblasts differentiate into different cell types or tissues, including endothelium, bone, smooth, cardiac or skeletal muscle, cartilage. Accordingly, the invention provides the use of mesoangioblasts for the preparation of differentiated tissues, for repairing damaged or diseased tissues, or in general for the treatment of pathologies that require tissue or cell repair, renewal or regeneration.

BACKGROUND OF THE INVENTION

It has been long believed that stem cells are only contained in tissues undergoing constant renewal, such as the epithelia and blood. In the last decade, also non-regenerating tissues, such as the nervous tissue, have been found to possess stem cells which indefinitely proliferate and generate neurons, oligodendrocytes and astrocytes, either from fetal or adult brain (Temple & Alvarez-Buylla, 1999; Gage, 2000). Neural stem cells, as well as the hematopoietic and mesenchimal stem cells, generate different tissues such as brain, blood or skeletal muscle, suggesting the existence of one or more plastic stem cells (Anderson et al., 2001).

The notion of plasticity of stem cells has greatly changed in the last years, since adult bone marrow has been demonstrated to contain progenitors that can form muscle fibers in the regenerating skeletal muscle (Ferrari et al.,

1998; Gussoni et al., 1999), epathocytes in the liver (Paterson et al. 1999; Lagasse et al. 2000) and neurons and glia in the central nervous system (Kopen et al 1999; Mezey et al. 1999; Brazelton et al. 2000). In addition, the central nervous system contains stem cells that can differentiate into hemopoietic cells (Bjorson et al. 1999) or into skeletal muscle (Galli et al. 2000).

The characteristics of the stem cells so far identified can be summarized as follows:

- Neural stem cells (NSC), propagating for long periods in culture, were isolated from both fetal and adult murine cerebral tissue and from fetal human cerebral tissue. These cells express specific markers (nestin) and grow like neurosphere in suspension and in specific serum-free medium containing FGF and EGF (Temple & Alvarez-Buylla, 1999; Gage, 2000). The same cells can differentiate into neurons, astrocytes and oligodendrocytes, as well as into blood and skeletal muscle (Bjorson et al., 1999; Galli et al., 2000). When grafted in a chick embryo Hensen's node, they differentiate into several cell types (Clarke et al., 2000).
- Mesenchimal stem cells (MSC) are established and expanded in culture from adult bone marrow stromal cells of different species, including humans (Bianco & Gehron Robey, 2000). They grow in substrate-adhered monolayers in serum-enriched medium and do not express endothelium and hematopoietic markers (Deans & Moseley 2000). They can be induced to differentiate in vitro and in vivo into cartilage, bone and adipose tissue. Their ability to differentiate into skeletal muscle has been recently reported (Reyes et al., 2001), but further clarification is needed in this respect. The inoculation of these cells in sheep embryos results in their incorporation in many tissues (Liechty et al., 2000).
- Hematopoietic stem cells have been in the clinical practice for decades

(e.g. for bone marrow transplantation). They are round-shaped, do not adhere to the substrate and can be cultured only in semi-solid medium or on feeder cell layers. These cells express distinctive markers (Flk+/Thy^{low}.Lin-/Kit+) that are not present in the bone marrow (Wright et al 2001), and can generate blood cells, neurons and glia (Brazelton et al. 2000, Mezey et al 2000), skeletal (Ferrari et al. 1998, Gussoni et al. 1999) and cardiac muscle and hepatic cells (paterson et al. 1999).

- Recently Multipotent Adult Progenitors (MAP) have been described as a sub-population of mesenchymal stem cells, growing indefinitely at low serum concentrations and capable of differentiating into virtually all tissues (Reyes, M. Lund, T. Lenvik, T. Aguiar, D. Koodie, L. and Verfaillie, C.M. (2001). Purification and ex vivo expansion of postnatal human marrow mesodermal progenitor cells. *Blood* **98**, 2615- 2625; Jiang, Y., Vaessen, B., Lenvik, T., Blackstad, M., Reyes, M. and Verfaillie, C.M. (2002). Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. *Exptl. Hematol.* **30**, 896–904; Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. (2002). Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 418, 41-49).

Besides the above adult stem cells, which are currently used in clinics, other embryonic stem cells or multipotent cell lines exist, the characteristics of which are outlined below.

The embryonic stem cells (ESC), isolated from blastocyst internal cells, are naturally immortal, pluripotent and able to colonize the germ line, but cannot be found in the embryo after implantation (Robertson et al., 1986; Evans, 1996; Gardner & Brook, 1997; Notarianni et al. 1991; Smith 2001). In

humans, these cells can only be isolated from embryos created for that specific purpose, by means of nuclear transfer from somatic cells to oocytes, or from embryos recovered from in vitro fertilization experiments.

Embryonic germ cells (EGC) are isolated from mouse fetal gonads, express typical antigens – similarly to hemopoietic stem cells – and can be expanded indefinitely in vitro (Donovan, 1994; Labosky et al. 1994). These cells are not able to colonize the embryo when injected into the blastocyst, nor their ability of generating different cell types has been so far demonstrated.

The search for the origin of pluripotent stem cells during fetal and perinatal development has recently revealed the existence of a sub-population of myogenic precursors in the dorsal aorta and in the somites, which represent the typical site where the skeletal muscle originates (De Angelis et al., 1999). In the same period Jackson et al. (1999) reported the presence of skeletal muscle progenitors that are able to regenerate the mouse hematopoietic system, thus revealing an unexpected relationship among different tissue progenitors, with an apparent common origin in the hemo-angioblastic system.

On the basis of these findings, it has been hypothesized that during tissue-histogenesis, when blood vessels penetrate the forming tissue, vessel-associated progenitors - which are likely generated by a common ancestor, such as a mesoangioblast or a hemoangioblast – leave the vessel and diffuse in the forming tissues where they enter a differentiation program (Bianco and Cossu 1999) depending upon local signals emitted by differentiating cells, which would be functionally different from the commitment of embryonic signaling centers such as the notochord and Hensen's node, even if the same molecules might be involved. Some of these progenitors may remain undifferentiated or they differentiate later during postnatal development or during regeneration. Because of their origin, these cells should maintain the differentiation potential when naturally or experimentally introduced in a

different tissue.

DESCRIPTION OF THE INVENTION

The transplantation of murine and quail embryonic or peri-natal aorta into chick at the embryonic or postnatal stage showed the presence of cell progenitors associated with donor vessels in several mesodermal tissues such as bone, cartilage, muscle, including tissues distant from the transplantation site, such as the myocardium. The aorta was able to generate a clonal progeny which could be indefinitely expanded in vitro while maintaining multipotency in vitro and in vivo. These observations suggested the existence of vessel-associated stem cells that in the fetal stage of development are distributed to forming tissues through angiogenesis and through the circulation.

This novel type of stem cell, termed "mesoangioblast", which is different from known stem cells, is able to generate most mesodermal tissues and can be isolated and expanded in vitro, according to the present invention, by the following steps:

a) providing vascular cells by dissociation of cultured embryonic, fetal, perinatal or adult mammalian vessels;

or

a') selecting ckit+ cells from postnatal or adult mammalian bone marrow or cord blood;

or

a'') selecting AC133+/CD34+ cells from the outgrowth of vessels within fragments of skeletal muscle tissue maintained in culture without changing the medium for a period of at least one week;

b) cloning the cells obtained from a), a') or a'') at limiting dilution on a feeder layer of Mitomycin C-treated primary fibroblasts;

c) selecting and growing positive clones and, optionally,

d) repeating culture passages on the feeder cell layer.

Following the above procedure, clonal populations of mesoangioblast stem cells are isolated.

In step c), Mitomycin C is preferably used at a concentration of 2.5 µg/ml for a period of 2 hours. In step d), the vascular cells are preferably cultured in 0.25 cm multiwell plates. Fibroblasts are preferably taken from E8-E10 embryos and passed twice or more in culture before being used as feeder layer for vascular cells; STO fibroblasts are preferred as they give the largest number of clones.

The vascular cells are prepared by enzymatic digestion (collagenase/dyspase) of explanted vascular tissue at different stages of embryonic, fetal or postnatal development. Although the stem cells isolated from postnatal tissues have the same characteristics as those derived from fetal tissues, their relative content is lower and therefore their cloning frequency is reduced. The suspension of single cells obtained by dissociation and digestion of the explanted vessel is cultured on the feeder cell layer. In step e), positive clones can be selected by previous labeling the dissociated tissues with a dye allowing for single cell detection, preferably with the fluorochrome DiI.

In a different embodiment (alternative (a') in the above general process), cells from cord blood or from bone marrow, expressing cKit as a surface antigen, are used in place of vascular cells as the source of mesoangioblasts. The mesoangioblast cell clones thereby isolated have the same characteristics as those of embryonic or fetal origin but they are less frequent and show a lower division rate.

In a further embodiment (alternative (a'')), human post-natal mesoangioblasts can be isolated from small vessels of skeletal muscle tissue from surgical biopsies. In this case, fragments (2 mm of diameter) of muscle tissue from biopsies are maintained in DMEM at 4°C for 1-5 days. Bundles of

3-5 muscle fibers, containing their associate microvessels, are then isolated from the larger fragments of the biopsy and placed in culture flask with medium containing DMEM + FCS and antibiotics. Culture flasks are incubated for a period of two to three weeks without changing the culture medium. This is important as the cells' own conditioned medium promotes the proliferation of mesoangioblasts within the culture. During this period, if necessary, fresh DMEM + FCS can be added to the culture medium. A morphologically heterogeneous population of cells containing 2-4% AC133+ (or Sca1)/CD34+ stem precursors is thus obtained. These latter have been identified and characterized as the mesoangioblast stem cells, which are isolated by cell sorting and cloned as above indicated. Further populations of mesoangioblasts may be continuously generated by the cell population left over from the cell sorting.

Regardless of their origin, the stem cells isolated according to the invention typically express the markers cKit, Flk-1 (Fetal Liver Kinase 1), Sca1 (Stem Cell Antigen 1), MEF2D (Myocyte Enhancer Factor 2), GATA-4, CD34 but fail to express either tissue-specific transcription factors such as Myf5, Nkx2.5 or Cbfa1 (Creb Binding Factor 1), or embryo stem cell specific transcription markers such as Oct-4.

Compared to the stem cells so far identified, the mesoangioblasts according to the invention display distinctive features:

- in contrast to NSCs, they do not show neurospherical growth and do not express nestin;
- in contrast to MSCs, they express hemoangioblastic antigens (cKit, Flk) and cannot be isolated with the same methods used for MSCs;
- in contrast to epithelial stem cells, they neither express epithelial antigens or genes, nor show epithelial morphology;
- in contrast to HSC, they adhere to the substrate and grow

indefinitely;

- in contrast to ES, they are present in the post-grafted embryo and do not express the ES marker Oct-4;
- in contrast to EGC, they are multipotent and negative for alkaline phosphatase – a marker of primordial germ cells;
- in contrast to MAP they express CD34, Flk-1 and c-Kit and can differentiate into mesoderm tissues only.

The above characteristics are found indistinctly in both human and animal pre- or post-natal cells.

A sample of murine mesoangioblastic stem cells has been deposited at the Centro Biotecnologie Avanzate (CBA, Italia) n. PD02005 on April 9, 2002.

Mesoangioblasts can be grown extensively in culture (more than 50 passages), yet they respect contact inhibition of growth, do not grow in soft agar and are not tumorigenic in nude mice assays. Different clonal lines of mesoangioblasts show similar though non identical growth and differentiation potential: when subjected to micro-array analysis, they show very similar profiles of gene expression among themselves and, interestingly, to those recently reported for hematopoietic, neural and embryonic stem cells. In keeping with their differentiation potential, mesoangioblasts express predominantly genes enriched in mesoderm some of which are receptors and signaling molecules for mesoderm inducing molecules such as BMP, Wnt and Notch.

The expression of several cytokines, such as VEGFB, FGF7, HDGF, PDGF and SDF1 (Fig. 10), in adult and embryonic mesoangioblasts, suggests a role for these stem cells in promoting angiogenesis in the host tissue and directly or indirectly promote survival and growth of host surrounding cells.

When injected into the blood circulation, mesoangioblasts accumulate

in the first capillary filter they encounter and are able to migrate outside the vessel, but only in the presence of inflammation, as in the case of dystrophic muscle. Indeed mesoangioblasts express many receptors for inflammatory cytokines and are able to migrate in vitro and in vivo in response to HMGB-1, a nuclear protein that is released by necrotic cells and acts as a potent inflammatory cytokine. In vitro, HMGB1 determines migration and proliferation of both adult and embryonic mesoangioblasts. In addition, HMGB1 also stimulates transmigration across an endothelial monolayer. Similar effects also occur in vivo: mesoangioblasts injected into the femoral artery migrated to the vicinity of HMGB1-coated heparin beads implanted into the tibialis anterior muscle, but were unresponsive to control beads. The identification of HMGB1 as a signal able to stimulate both stem cell motility and proliferation suggests a potential approach to improve and control tissue regeneration.

The intra-arterial delivery of wild type mesoangioblasts corrects morphologically and functionally the dystrophic phenotype of virtually all downstream muscles in adult, immunocompetent alpha sarcoglycan null mice (a model for limb-girdle muscular dystrophy). When mesoangioblasts, isolated from juvenile dystrophic mice and transduced with a lentiviral vector expressing alpha sarcoglycan, were injected into the femoral artery of dystrophic mice, they reconstituted skeletal muscle similarly to wild-type cells. In comparison with skeletal myoblasts, mesoangioblasts show the ability to cross the endothelium and migrate extensively in the tissue interstitium where they are recruited by regenerating muscle fibers thus reconstituting the dystrophin-glycoprotein complex. The success of this protocol was mainly due to widespread distribution of donor stem cells through the capillary network, a distinct advantage of this strategy over previous approaches.

Under appropriate conditions the mesoangioblasts can differentiate into

different cell types, including endothelium, especially monocytes and macrophages, osteoclasts, smooth muscle, cardiac and skeletal muscle, cartilage and bone tissues. The differentiation process will vary depending on the desired cell-type but in general it will comprise exposing mesoangioblasts to growth factors, vitamins, hormones, cytokines, or their co-culture with cells from a differentiated tissue. The end of the differentiation process can be determined by the activation of at least two specific genes of the differentiated tissue and/or by the acquisition of functional or morphologic characteristics thereof. In the case of skeletal muscle tissue, for example, the expression of myogenic markers such as Myf5 or MyoD or the development of sarcomers and of contractile activity are differentiation indicators.

Accordingly, the mesoangioblast stem cells of the invention can be used in the preparation of differentiated tissues, e.g. for restoring the structural and functional integrity of damaged tissues, especially for curing diseased tissues, more generally for the treatment of pathologies that require tissue or cell repair, renewal or regeneration. Typical clinical indications include, but are not limited to, muscular dystrophies (e.g. limb-girdle muscular dystrophy) and myocardial infarct. For use in therapy, mesoangioblasts can be combined with suitable carriers, supports, vehicles or excipients, or with different biologically active substances, such as IGF-I or myostatin inhibitors for the treatment of myocardial injury.

Furthermore, the mesoangioblasts can be genetically modified to deliver therapeutic genes for tissue or cell-specific gene replacement or for treating genetic defects. In particular, wild-type or genetically modified mesoangioblasts can be expanded in vitro and directly delivered in large numbers through the arterial circulation.

Mesoangioblasts can also be used for the identification of genes and proteins involved in the differentiation process. For example, in vitro assays,

preferably large-scale screening assays such as microarrays, proteomics or antibody libraries-based assays, aimed at identifying the molecules relevant to tissue-specific differentiation, especially the differentiation into cardiac or other mesodermal tissues, or to the maintenance of stemness, can be set up using mesoangioblast cells or isolated components thereof.

DESCRIPTION OF THE FIGURES

Figure 1 – isolation of murine embryo mesoangioblasts

A: Morphology of the embryonic dorsal aorta isolated from E9.5 mouse embryos after pancreatin digestion. Note the absence of remnants of adjacent embryonic structures.

B: RT-PCR revealed that endothelial and hematopoietic markers (VE-Cad, FLK1 and CD34), but not myogenic markers (Myf5 and MyoD), were expressed in dissected aorta (A); total embryo cDNA was used as a positive control (+). Negative control (no RNA) is shown in the first lane (-).

C: Phase contrast morphology of a clone from embryonic aorta growing on a STO feeder layer.

D: Phase contrast morphology of one typical clone (A4) from embryonic aorta after five passages in vitro.

E: RT-PCR of the messages expressed by several cell lines (A4, A6, A14 and B13) from the dorsal aorta after 5 passages in vitro. Note expression of hemo-angioblastic but not of tissue specific markers such as Myf5 or Nkx2.5.

Figure 2 – isolation of postnatal murine mesoangioblasts

A: Bone marrow from which the initial population can be isolated.

B: Large/medium vessel surgically isolated.

C: FACS separation of c-Kit+ cells.

D: Blood vessel fragment explantation.

E: Morphology of a newly formed mesoangioblastic clone.

F: Morphology of adult murine mesoangioblasts after two passages in culture.

Figure 3 – isolation of human mesoangioblasts

A, B, C: 6-weeks human fetus (A) from which the aorta-gonad-mesonephro region (AGM, B) and then the aorta are isolated and, respectively, dissected (C)

D: Morphology of a human mesoangioblastic clone.

E: Morphology of human mesoangioblasts after two passages in culture.

Figure 4 – In vitro differentiation of the aorta derived cell line A4 into different cell types.

A: Smooth muscle cells (SMA positive, arrowheads).

B: Osteoblasts (ALP positive, arrowheads) are detected after treatment with 1 ng/ml BMP2.

C: Adipocytes are detected after treatment with 10 ng/ml of dexamethasone.

D: Skeletal myotubes are detected after co-culture of GFP-labeled A4 cells with C2C12 myoblasts. Cells expressing both GFP and myosin heavy chains (a marker for myotubes) are indicated by the arrowhead in the merged image. These represent A4 cells differentiated into myocytes. A mononucleated, differentiated, GFP-positive myocyte is shown in the inset in D.

E: Cardiocytes are detected after co-culture of GFP-labeled A4 cell with rat neonatal cardiocytes. Cells expressing both GFP and cardiac specific troponin 1 (a marker for cardyocyte differentiation) are indicated by the arrowhead in the merged image. These represent A4 cells differentiated into cardiomyocites.

Figure 5 – Chick-mouse chimeras (sacrificed at E14) transplanted with mesoangioblasts of clonal cell line A4

A: Whole-mount fluorescence of the transplant site, showing a cluster of GFP+ donor cells.

B: Section through a large vessel showing donor cells integrated into a large vessel wall (arrowhead) stained for smooth alpha-actin.

C: A cluster of donor cells outside a small vessel, with one cell (arrowhead) double expressing GFP and SMA. Inset: GFP circulating cells inside a small vessel (arrowhead).

D: Donor cells accumulate in the dermis (arrow) bur not in the epidermis (arrowhead, to the right of the dermis in panel) or in the muscle primordium (to the left of the dermis in the panel).

E: Section through the myocardium showing several donor cells in the myocardium (arrowhead) and in the sub-epicardium.

F: Section through the intestine showing donor cells in the smooth muscle layer (arrowhead) stained for SMA

G: Section through axial tissues showing donor cells in the cartilage adjacent to the perichondrium (arrowhead), in the perichondrium (asterisk) and in the blood vessel (arrow), bur not in the intervening muscle fibers.

H: Section through a muscle primordium. Arrowhead indicates fibers double-labeled with GFP and myosin heavy chain. These are fibers derived from donor cells.

Figure 6 - Expression of α-SG and of other dystrophin associated proteins in α-SG null mice after intra-arterial delivery of wt mesoangioblasts.

A: Low magnification of the untreated Quadriceps (upper lane), of the Quadriceps (middle lane) and the TA (lower lane) of α-SG null mice that were injected with wt mesoangioblasts 2 months before sacrifice. Large areas of the

treated muscle expressed α-SG after staining with a specific antibody (the fluorescent labeling appears in a lighter shade of colour. Sections were also stained with anti-laminin antibodies and Dapi.

B: Higher magnification of Soleus from the same treated animals revealed extensive reconstitution of the dystrophin complex after double staining with antibodies against laminin (lighter shade of color in the upper panel) or sarcoglycans and dystrophin (dy) (lighter shade of color in the lower panel).

C: Western blot analysis of proteins isolated from a post-nuclear membrane fraction of two large muscles (Quadriceps and Gastrocnemius) from α-SG null mice after 30, 60 or 90 days from the injection of wt mesoangioblasts. The filters were reacted with antibodies directed against α-SG (11); MyHC (3) and β1 integrin are shown as internal controls.

Figure 7 - Functional properties and cross sectional areas of individual muscle fibers of long term treated α-SG null dystrophic muscles, after three consecutive injections of wt mesoangioblasts

Specific tension (Po/CSA), maximum shortening velocity (Vo), and distribution of cross-sectional areas (CSA) were measured in a population of 188 single muscle fibers isolated from gastrocnemius muscles of CTR mice (n=2), α-SG null mice (n=2), and α-SG null-treated mice (n=2). The experimental approach and the solutions used to determine CSA, force (Po) and Vo of isolated muscle fibers have been previously described in detail (17,18). As in all animals studied the large majority of fibers (~80%) from Gastrocnemius muscles contained MyHC-2B only type 2B fibers were used for comparison and reported in A and B.

A: The mean values of Po/CSA (filled bars) and of Vo (empty bars) of type 2B single skinned muscle fibers from the three groups of mice: CTR (black bars), α-SG KO (red bars) and α-SG KO, treated (blue bars).

B: The distribution of CSAs in the three populations of fibers. The box & whiskers plot shows the median of CSAs or 50th percentile (indicated by the line in the middle of the box), the 75th percentile (i.e. the top of the box), the range of CSAs (i.e. the width of the box) and the smallest and the largest CSAs (i.e. the top and bottom of the whiskers).

C: The distribution of CSA values of centrally nucleated, i.e. regenerating, muscle fibers from α-SG KO (upper histogram, red bars) and αSG KO-treated mice (lower histogram, blue bars) determined on hematoxylin and eosin stained cross-cryosections of bundles of Gastrocnemius muscles.

Figure 8: - Morphology of long term treated α-SG null dystrophic muscles, after three consecutive injections of wt mesoangioblasts.

A: Haematoxilin-Eosin staining of the Soleus of a 6 month-old control wt (CTR) and similarly aged α-SG null animals (α-SG KO) and α-SG null, treated mice (treated). Higher magnification of the sections are also shown. The untreated dystrophic muscle showed a large area of necrosis and disrupted morphology that was significantly reduced after treatment.

B: Evans Blue dye injection in the tail vein: red identifies damaged fibers that have uptake the die; nuclei are revealed in blue by Dapi staining. (C) Azan-Mallory staining: extra-cellular scar tissue stains with this dye.

Figure 9 - In vitro gene transfer into dystrophic mesoangioblasts and tissue replacement with transduced cells.

A: Western Blot analysis of packaging 293T (at 72, 48 and 16 h after transfection) and α-SG null mesoangioblasts transduced with a third generation lentiviral vector expressing α-SG cDNA under the transcriptional control of the PGK promoter and followed by an IRES-GFP, reacted with a monoclonal antibody against α-SG (sk mu) α-SG from skeletal muscle.

B: Immunofluorescence analysis of C2C12 myotubes transduced with the vector and stained with anti-MyHC antibody (left panel). Transduced

cells also expressed GFP (middle panel). Double stained cells are shown in the right panel.

C: Immunofluorescence analysis of a co-culture of transduced mesoangioblasts with control C2C12, showing a large number of MyHC/GFP positive myotubes, that incorporated GFP positive mesoangioblasts.

D: Immunofluorescence with anti α-SG antibodies of the Gastrocnemius muscle injected 3 times with 5 x 105 dystrophic, transduced mesoangioblasts through the femoral artery revealed many α-SG /GFP positive fibers. Double staining with α-SG and anti-laminin antibodies is also shown in lower images.

E: Specific tension (Po/CSA) of single muscle fibers of Gastrocnemius muscles from CTR mice (left bar), α-SG null mice (middle bar) and α-SG null mice treated with dystrophic, transduced mesoangioblasts (right bar).

Figure 10 - PCR profiles of expression of cytokines in mesoangioblast cell lines.

The panel shows level of expression of VEGFB, FGF7, HDGF, PDGF A and SDF1 in different cell lines as visualized by SEMI-QUANTITATIVE RT-PCR amplification of total RNA after 30 cycles (VEGFB, FGF7, HDGF,PDGFA) and 35 cycles (SDF1). fibro: fibroblasts Sat pro: Satellite cells (myogenic)proliferative Sat dif: Satellite cells (myogenic) differentiated D16: mesoangioblasts 44B: ES derived endothelial cells G1: adult mesoangioblast Lin-: hemopoietic fraction enriched with stem cells E 9.5: total mouse embryo at 9.5 days of developemnt HDGF: Hepatoma derived growth factor SDF1: Stroma derived growth factor 1

VEGFB is expressed at high levels in embryonic and adult mesoangioblasts (D16 and G1) in panel 1 as well as in endothelial cells derived from embryonic endothelium (44B) FGF7 is expressed in all the cell lines at approximately the same level with a marginal increase in embryonic and adult

mesoangioblasts (D16 and G1) HDGF expression is absent form Lin- cells but is present in the other cell lines; PDGFA, while not present in 44B and Lin- is highly expressed in embryonic and adult mesoangioblsts and SDF1 is absent from 44B but present at low levels Lin- cells but it is highy expressed in adult mesoangioblasts (G1).

EXAMPLE 1 – isolation, characterization and in vitro differentiation of mesoangioblasts

Methods

Transgenic mouse lines

Experiments were performed using embryos derived from wild type or from MLC1/3F-nlacZ (Kelly et al., 1995) transgenic mice. In the latter the bacterial beta-galactosidase reporter gene is under transcriptional control of the low chain 1/3F myosin promoter/enhancer, which restricts the expression to skeletal muscle.

Transplantation studies

Mouse cells (approximately 10^4 cells) were transplanted into a slit made between neural tube and somite at the thoracic inter-somitic levels in a E2 (HH 12 to 14) chick embryo (Fontaine-Perus, 2000).

Flow cytometry

In some experiments, the aorta was digested for 30 minutes with 0.1% collagenase and isolated cells were incubated at 4°C for 1 hour with anti-Quek 1, anti-QH1 (quail) or anti-PECAM (mouse) monoclonal antibodies (supernatant diluted 1:10), and were washed and reacted with FITC-conjugated anti-mouse IG (Cappel, 1:300). After three final washes, cells were separated on a FACS scan using a Consort-30 software. Forward and 90°C side-scatter were used to identify and gate positive and negative fractions. Background subtraction using an unrelated antibody was performed for each sample. Approximately 10^4 cells for each fraction were centrifuged and then

implanted as pellets into recipient embryo.

Cell culture and clonal selection

Quail or mouse embryo fibroblasts were isolated from trypsin digestion of E3 quail and E10 mouse embryos and passed twice in culture before being transplanted as described above or used as feeder layers (after Mitomycin C treatment: 2.5 μg/ml for 2 hours in complete medium).

Mouse dorsal aorta from E9.5 embryos was grown as an explant culture as described elsewhere (De Angelis et al., 1999). Five days later the explant was dissociated into a single cell suspension, labeled with DiI as described (Tajbakhsh et al., 1994). After labeling, an aliquot of cells was stained with Hoechst to confirm that more than 95% of the cell population had been labeled. The cell suspension was plated at limiting dilution on a feeder layer of Mitomycin C-treated primary embryonic mouse fibroblasts, or STO fibroblasts in 96 multiwell plates in complete medium. After 6 hours, the plates were scored under an inverted fluorescence microscope and only wells containing 1 DiI labeled cell were considered for further analysis. After 1 week, clones appeared in approximately 2-4% of the wells. When the clones had grown to approximately 10^3 cells they were passed twice on feeder layers and thereafter on gelatin-coated dishes. Among different feeder layers, STO fibroblasts consistently gave the largest number of clones and were used in all successive experiments. To date, the clonal isolates have been grown in culture for more than 50 passages with a doubling time of about 12 hours. They have been sub-cloned with a cloning efficiency of approximately 10%. Attempts to expand the clones without feeder layers and with various growth factors (e.g. bFGF, PDGFbb, VEGF, IGFI, EGF, LIF, IL3) in different concentrations and combinations have been unsuccessful. Among the different clones initially characterized, clones A4 and B13 were used for the in vitro and in vivo differentiation experiments described here. Other clones are

shown in the RT-PCR analysis.

Isolation of mesoangioblasts from human fetuses

The same procedure is applied to human fetus explants with a pregnancy age of 5 to 10 weeks. The results are similar, except that human cells have a reduced growth by one division every 36 hours.

Isolation of mesoangioblasts from postnatal tissues

Large and medium-sized blood vessels (aorta, femur, popliteus, etc.) have been isolated from mice variously aged from 1 to 60 days. The tissue is mechanically separated from the surrounding connective under dissection microscope; fragments of around 1.2 mm^3 are explanted in vitro as described. The procedures are the same as described for fetal vessels. Generally the cloning frequency is about 100 fold lower than that observed with fetal vessels.

In vitro differentiation of A4 and other clonal isolates from dorsal aorta

Cells were treated with 1 ng/ml of BPM 2 in complete medium for 5 days (BPM2 was added every other days), then fixed and stained for alkaline phosphatase or analyzed for the expression of osteogenic markers by RT-PCR. Alternatively, cells were treated with 10 ng/ml of dexamethasone under the same conditions, and then analyzed for adipocyte morphology and expression of adipogenic markers. To induce osteoclastic differentiation, A4 cells were treated with 10^{-8} M 1,25 (OH)2 vitamin D3 for 5 days. For co-culture experiments, cells were infected for 4 hours with third generation lentiviral vector pRRLsin.PPT-PGK.GFP expressing green fluorescent protein (GFP) as described elsewhere (Follenzi et al., 2000). Approximately 80-90% of the infected population expressed GFP in the cytoplasm. GFP+ cells were cultured with a fourfold excess of unlabeled C2C12 myoblasts or rat neonatal cardiocytes, or with a 10-fold excess of adult bone marrow cells in Dexter-like conditions as described previously (Dexter and Testa, 1976). After different

periods, cultures were analyzed for co-expression of GFP and tissue-specific markers (MyHC, cardiac troponin 1 and CD45) by either immunofluorescence or FACS analysis.

Immunocytochemistry

The following antibodies were used in this work: QCPN monoclonal antibody (which recognizes quail but not chick nuclei) was obtained from the Developmental Studies Hybridoma bank under contract number NO1-HD-2-3144 from the NICHD; anti-Queckl and anti-QH1 monoclonals (which recognize the quail VEGF receptor and endothelial cells, respectively,were kindly donated by Anne Eichmann (Eichmann et al., 1997) and by Luc Pardanaud (Pardanaud et al., 1987) (Institut d'Embryologie, Nogent sur Marne, France); anti-miosin heavy chain polyclonal antibody (which recognizes all vertebrate sarcomeric myosins) (Tajbakhsh et al., 1994) was produced in the laboratory; anti-cardiac troponin 1 monoclonal antibody (Di Lisi et al., 1998) was donated by Stefano Schiaffino, University of Padua; anti-a smooth muscle actin monoclonal was from Signet, Dedham, MA; PE-conjugated anti CD-45, anti-Ter119, anti-Mac-3, anti CD11b and anti-Gr-1 were from Pharmingen, BD; anti-PECAM rat monoclonal was a generous gift from A. Vecchi (Istituto Mario Negri, Milan, Italy). Single and double immunolabeling, and alkaline phosphatase cytochemistry on paraffin wax-embedded or cryostat sections of chick chimeras were performed as described elsewhere (Tajbakhsh et al., 1994; Bianco et al., 1993).

RT-PCR

RT-PCR was performed as described previously (Ferrari et al., 1997). The oligos used for amplification of the VE-Cad, Flk1, CD34, Myf5, MyoD, Cbfa1, Kit, Nkx, Mef2d, Oct4 genes and the calcitonin receptor gene are described in Minasi et al., Development 129, 2773-2783 (2002).

RESULTS

Isolation and characterization of clones from mouse embryos, and their expansion in culture

The commonly accepted criteria for the identification of stem cells are self-renewal properties and the ability to generate differentiated cell types at any time. To investigate whether the population of multipotent progenitors from dorsal aorta actually contain stem cells, explants from E9.5- and E10.5-embryo aortas were cultured for 5 days, dissociated into single cells and these were cloned by limiting dilution on a layer of Mitomycin C-treated STO feeder cells. Cell-labeling with DiI allows for the identification of single cells in each plate-well. After 1 week, positive clones (2-4 every 96-well plate on average) contain cells highly refractive surrounded by cells with a tile-type morphology (Fig. 1 A). After two passages in culture, most of the clones continued to grow independently of the feeder cells and could be maintained in culture for more than one year. The cells have a division time of around 12 hours, maintain the morphology illustrated in Fig. 1 D and do not show senescence. Either immediately after their isolation, or after repeated expansion in vitro, all of the clones express cKit, Flk-1 and MEF2D, but they fail to express either tissue-specific transcription factors such as Myf5, Nkx 2.5, Cbfa1 or transcription factors specific for embryo stem cells such as Oct-4 (Fig. 1 E).

To test whether the mesoangioblastic stem cells are also present in postnatal or adult individuals, the same experiments were carried out on i) vessel fragments isolated from adult mice, explanted for 7 days and then cloned as described above (Fig. 2B), ii) a subpopulation of bone marrow cells (Fig. 2A). The vessel fragments produce fibroblast-like cells (Fig. 2D), some of which are positive for SMA (smooth muscle). The nucleated bone marrow fraction is incubated with an antibody against c-Kit (Fig. 2C), and then

positive cells are selected and cloned on feeder cells. In both cases, after 5 days small clones appear, which are similar to those derived from the embryo (Fig. 2E) and can be expanded for at least ten passages in culture. In particular, these clones show the same morphology (Fig. 2F), gene expression and differentiation potential as the embryonic mesangioblasts.

Two major differences are observed: 1) the clones obtained from adult vessels are about 100 times less frequent than those obtained from embryo vessels using explants of the same size; 2) the mean division time is about two times higher for adult mesangioblasts (24hrs).

To test whether mesangioblastic stem cells are present also in humans, the same experimental scheme was applied to i) vessel fragments isolated from human embryos aged 6 to 12 weeks (Fig. 2A-C), ii) vessel fragments isolated from human umbilical cord; iii) c-Kit+ cells from cord blood. The results of the experiments using human and murine tissues are identical. It was observed that the number of clones obtained is inversely proportional to the donor age. Human cells grow slower than their murine counterparts and show a division time of about 36-48 hrs.

Differentiation of mesangioblasts into different cell types

The cell lines derived from aorta explants were analyzed for the expression and maintenance of the multipotency found in freshly isolated cells. To this end, the cells were cultured with either specific cytokines known to induce a differentiation pathway or with in-vitro differentiating cells, to reproduce the micro-environment where mesoangioblasts are naturally delivered during in vivo angiogenic processes in fetal tissues. Figure 4A shows that the cells of the A4 clone, as well as other examined clones, contained approximately 10% smooth muscle actin (SMA)-positive cells. Each subclone of the A4 clone contained approximately 10% of SMA-positive cells. Experiments of BrdU incorporation indicated that SMA-positive cells have a

lower division rate than the whole population. A possible explanation for these results is that pericytes SMA+ are spontaneously generated by mesoangioblasts but their relative amount remains unvaried because of their lower division rate. However, if the cells are treated with TGF-beta, up to 50% mesoangioblasts convert to pericytes SMA+. When treated with BMP2, A4 cells express osteoblast markers including alkaline phosphatase, bone sialoprotein and Cbfa1 (Fig. 4B). When treated with dexamethasone, A4 cells differentiate into adipocytes (Fig. 4C). Alternatively the cells can be transduced with lentivirus vectors expressing GFP and then co-cultured with non-transduced skeletal or cardiac myoblasts. After five day culture, the co-expression of GFP and of skeletal muscle-specific antigens indicates that part of A4 cells have differentiated into either skeletal muscle (Fig. 4D) or cardiac (fig. 4E) cells. In addition the cell population contained a small fraction (3-5%) of CD45 positive cells.

EXAMPLE 2 – cell therapy of alpha sarcoglycan null dystrophic mice through intra-arterial delivery of mesoangioblast

Restoration of α-sarcoglycan and dystrophin-glycoprotein complex by wild type mesoangioblasts. We next investigated whether injection of wt mesoangioblasts in the muscles of α-SG null mice may restore expression of α-SG and of the whole dystrophin complex (55). Two months after a single injection of 5 x 105 wt mesoangioblasts (n=4), many areas of the Quadriceps, Tibialis Anterior, Soleus, Gastrocnemius and EDL expressed α-SG whereas the protein was completely absent from the muscles of the null mouse (Fig.6A). All the muscle fibers that expressed α-SG after mesoangioblast injection, also expressed β, γ, δ sarcoglycans and dystrophin (an example is shown for the Soleus in Fig. 6B). Western blot analysis confirmed the presence of the α-SG protein in skeletal muscle of α-SG null mice treated with wt mesoangioblasts. Protein expression was comparable with wt muscles

in membrane fractions of either Gastrocnemius or Quadriceps from treated animals (Fig. 6C). A similar pattern was maintained at least up to 3 months after a single mesoangioblast injection. Comparable levels of integrin β1 subunit, and myosin heavy chains (MyHC) were detected in all the different samples analyzed (Fig. 6C). Taken together, these results show that intra-arterial mesoangioblast delivery was effective in restoring expression of α-SG protein and of the other members of the dystrophin-glycoprotein complex in treated α-SG null mice. No immune reaction occurred against reconstituted fibers, even though low titer serum antibodies to α-SG were detected in treated mice.

Long term effect of donor wild type mesoangioblasts.

We then injected 5 x 105 male wt mesoangioblasts three times (at 40 day intervals) into the femoral artery of two month old α-SG null female mice (n=3). Animals were analyzed 4 months after the first injection (at 6 months of age). The histological analysis of skeletal muscle tissue of animals treated by three injections, showed an increased number of apparently normal fibers and reduction of the necrotic areas and of cellular infiltrates (Fig. 7A). Consistent with the histology, immunofluorescence analysis revealed the widespread presence of α-SG throughout the whole Soleus muscle (more than 50% of the fibers) in contrast with the total absence of signal in untreated α-SG null mice. We found markedly decreased uptake of Evans Blue dye in skeletal muscle of α-SG null mice treated with mesoangioblasts as compared with untreated animals (Fig. 7B), demonstrating a preserved integrity of the sarcolemma (55). Finally, staining with Azan Mallory revealed a marked reduction of the fibrosis (stained in blue by the dye) in the muscles of treated animals (Fig. 7C).

Complete functional recovery of treated muscle.

The physiology of hind limb muscles was studied in six month-old

α-SG null mice treated with three injections of wt mesoangioblasts and the results obtained indicate that the amelioration of skeletal muscle morphology sustains a recovery of function. Previous studies have suggested that functional analysis of whole skeletal muscles in vitro is not a sensitive approach to show impairment of skeletal muscle function in α-SG null mice. In fact, notwithstanding a clear impairment in animal motility, tetanic force of whole muscles of α-SG null mice was not significantly decreased (50). To better assess the skeletal muscle function, we dissected a large population (n=188) of individual muscle fibers from Gastrocnemius muscles of control, α-SG null and mesoangioblast-treated α-SG null mice, and measured cross-sectional area (CSA), specific force (Po/CSA) and maximum shortening velocity (Vo) (Fig 8A and B). All fibers used were identified on the basis of myosin heavy chain isoform (MYHC) composition (56,57). As the large majority of fibers contained MyHC-2B (~80%), only type 2B fibers were used for comparison. The analysis of the distribution of CSAs in the three populations of fibers revealed an increase in fiber size in α-SG null mice in comparison with control mice, and a partial recovery of normal size in mesoangioblast-treated α-SG null mice (Fig. 8B). The median of CSAs was higher for α-SG null mice (5075 μm2) than for controls (3369 μm2) and intermediate for mesoangioblast-treated α-SG null mice (4060 μm2), and a similar trend was clear also for the 75th percentile (8196 μm2 for α-SG null mice, 4904 μm2 for control mice, and 5957 μm2 for mesoangioblast-treated α-SG null mice). Finally, both the range of CSAs between the 25th and 75th percentile, and the smallest and the largest CSAs strongly suggest that the range of variability of CSAs was larger for α-SG null mice than for controls and intermediate for mesoangioblast-treated α-SG null mice. Po/CSA was significantly lower ($p<0.001$) in single muscle fibers from α-SG null mice (43.63(23.26 SD kN/m2, n=78,) than in single muscle fibers from controls

(60.76(22.70 kN/m2, n=50). Po/CSA significantly ($p<0.001$) recovered to normal values in mesoangioblast-treated α-SG null mice (65.44(26.66 kN/m2, n=60). No difference was found in Vo among the three fiber groups. As all fibers were type 2B fibers, the latter result is fully consistent with the well known dependence of Vo on MyHC isoform composition (58). The lack of difference for Vo suggests that neither muscular dystrophy nor mesoangioblast treatment affected the kinetics of acto-myosin interaction (59). Morphometric analysis of centrally nucleated (i.e. regenerating) fibers indicated that treated animals had a size distribution different from untreated dystrophic mice (Fig. 8C). Large, degenerating fibers were absent and a single mode distribution comparable with normal fibers was observed, suggesting that once regenerating fibers incorporate mesangioblasts they are less susceptible to further degeneration. Furthermore, we counted the total number of fibers in soleus muscles from control (n=2), α-SG null (n=2) and mesoangioblast-treated α-SG null mice (n=2) mice. Cross-cryosections of soleus muscles from the right leg (treated leg in α-SG null-treated mice) were stained with haematoxylin and eosin. Care was taken to cut sections that extended till the edge of muscles. Two sections from the portion of the muscle with the largest CSA were chosen for analysis. A significant decrease ($p<0.05$) was observed in α-SG null mice (236.33(37.30) in comparison with CTR (378.00(30.40). A trend towards a recovery of total fiber number was observed in mesoangioblast treated α-SG null mice (319.00(26.87) suggesting a more effective regeneration of muscle fibers. At 6 months of age, dystrophic animals show reduced motility in the cage; in contrast, treated animals maintained a certain degree of spontaneous motility despite a mono-lateral treatment. To quantify the extent of residual motility, control, dystrophic and mesoangioblast-treated dystrophic mice (2 animals for each group) were tested by forced run on the rotarod (60) at a fixed speed (1.6 m/min for 4 minutes; 2

minutes run plus 1 minute rest plus 2 minutes run). On average, the control animals fell off the rotarod only 4 times in the 4 minutes test and never stopped running before the end of the test. The α-SG null mice could hardly run on the rotarod, i.e. fell of 13 times per minute and stopped running after 1 minute. The mesoangioblast-treated α-SG null mice fell off 10 times per minute and could run for 3 minutes before stopping. These data strongly suggest that functional recovery of treated muscles led to better motility, despite the fact that only one leg had been treated.

Morphological and functional recovery by autologous, genetically corrected mesoangioblasts.

To test whether autologous, genetically corrected stem cells may represent a possible model for the therapy of muscular dystrophy, we isolated mesoangioblasts from vessels of juvenile dystrophic mice (15 days). Mesoangioblasts are obtained at much lower frequency from adult than from embryonic vessels and grow at a lower rate (division time 24 vs 12 h) but they are equivalent to their embryonic counterparts in terms of gene expression and differentiation potency (43). We produced a third generation lentiviral vector (52) expressing the mouse α-SG cDNA and GFP cDNA as a reporter gene, hPGK-GFP-αSG vector. After 48 hours of virus assembly in 293 T packaging cells, we collected the supernatant containing the viral particles and used it to infect mesoangioblasts at an MOI of 200 for 24 hours. Under these conditions, more than 90% of the cell population was efficiently transduced. Furthermore, 293 T cells as well as α-SG null transduced mesoangioblasts efficiently produced the recombinant protein (Fig. 9A) at a level comparable with normal skeletal muscle (22), a key requirement to prevent α-SG dependent cytotoxicity. When C2C12 myoblasts were transduced with this vector more than 90% of the population co-expressed GFP and MyHC upon differentiation (Fig. 9B); similarly α-SG null mesoangioblasts, transduced with the vector,

efficiently differentiated into muscle cells when co-cultured with uninfected C2C12 cells and co-expressed GFP and MyHC (Fig. 9C). Transduced mesoangioblasts did not change their growth rate and did not acquire features of transformed cells. Four months after three intra-arterial injection of 5 x 105 αSG transduced dystrophic mesoangioblasts, many fibers expressed a strong cytosolic diffuse GFP signal and α-SG on the membrane (Fig. 9D). After long term treatment with genetically corrected mesoangioblasts, α-SG null mice had restored specific force in individual fibers of their Gastrocnemious muscle (Fig. 9E) and also showed an ameliorated motility on the rotarod test, similar to the effect observed with dystrophic mice treated with wt mesoangioblasts. No immune reaction was detected against the GFP protein although low titer antibodies were present in the serum. Indeed, immunostaining with antibodies against MAC-1, that identifies infiltrating macrophages, revealed reduced number in the muscles of treated α-SG null mice.

Discussion

These data indicate that mesoangioblasts represent a novel promising approach towards the cell therapy of primary myopathies. In comparison with skeletal myoblasts, mesoangioblasts show the ability to cross the endothelium and migrate extensively in the tissue interstitium where they are recruited by regenerating muscle fibers thus reconstituting the dystrophin-glycoprotein complex. While this is also the fate of blood born progenitors from the bone marrow (62,66), the frequency of this event is too low to result in significant amelioration of the dystrophic phenotype (63,66). In contrast mesoangioblasts can be expanded in vitro and directly delivered in large numbers through the arterial circulation, with no need for a complex procedure such as bone marrow transplantation. Intra-arterial injections are simple and safe procedures in patients where they may be repeated frequently, in contrast to the mouse where a surgical procedure is required.

In comparison with mesoangioblasts, viral vectors do not cross the endothelium and require intra-muscular delivery: they transduce muscle fibers very efficiently but only in the injected area due their limited diffusion (67,68). Finally, a number of studies indicate amelioration of the dystrophic phenotype by expression of biologically active molecules such as IGF-I or neutralizing antibodies for myostatin (69,70). These strategies that do not lead to gene replacement, may be part of a future combinatorial therapy by preserving muscle integrity and thus improving the efficacy of cell therapies.

EXAMPLE 3 – gene expression profile

We have analyzed by micro-array analysis the profile of gene expression of four independent clonal derived lines of mesoangioblasts in comparison with a line of embryonic fibroblasts. The results obtained show that the profile of genes expressed by the four mesoangioblast line are more similar to each other than to the fibroblast line, although they still differ among each other. They largely overlap with published sequences for hematopoietic, neural and embryonic stem cells.

Among families of known genes, many pathways of response to developmental signaling molecules are activated: among these Wnt, Frizzled, Dishevelled and Tcf are all expressed suggesting the existence of an autocrine loop for proliferation and indeed forced expression of Frzb-1 inhibits cell division. In contrast genes of the Sonic hedgehog pathway are not expressed and this may explain the inability of these cells to activate myogenesis in response to Wnt and Shh, as it happens in embryonic myogenesis. In addition pro-osteogenic genes are highly expressed and in fact these cells are efficient in forming bone in response to BMP for which they express receptor and genes of the signal transduction pathway. This is not a general rule since mesoangioblasts express many pro-neural genes and yet undergo only abortive neurogenesis under various conditions that promote it. This phenomenon

cannot be ascribed to absent expression of neural regulatory genes, since forced expression of neurogenin 1 or 2, MASH or NeuroD also failed to induce neurogenesis, probably implying a refractory state to neural differentiation pathways. Finally mesoangioblasts express a number of pro-inflammatory genes, cytokines and cytokine receptors, similarly to what observed in other types of stem cells.

Together, these data define a unique phenotype for mesoangioblasts, explain several of their biological features and set the basis for future functional studies on the role of these cells in tissue histogenesis and repair.

EXAMPLE 4 – extracellular HMBB1 induces mesoangioblast migration and proliferation

Materials and methods

Cells

Bovine Aorta Endothelial Cells (BAEC) were isolated from a section of the thoracic aorta of a freshly slaughtered calf as described.

Mesoangioblasts were isolated from the dorsal aorta of mouse embryos and from juvenile arteries as previously described. After cloning, cells were expanded on a feeder layer of mitomycin C-treated STO fibroblasts. Clones showing the mesoangioblast gene expression pattern (presence of CD34, Kit, Flk1 and MEF2D) were used for the in vitro and in vivo experiments. Embryonic mesoangioblasts (clone D16) were transduced with a lentiviral vector encoding for nuclear LacZ, while adult mesoangioblasts (clone G1) were labeled with DiI and then injected into the femoral artery of mice.

HMGB1 and anti-HMGB1 antibodies

Expression and purification of the full-length HMGB1 protein and fragments thereof was performed as described previously (Müller, S., Bianchi, M. E. & Knapp, S. Thermodynamics of HMG1 interaction with duplex DNA. *Biochemistry* **40**, 10254-10261 (2001).

Endotoxins were removed by passage through Detoxy-Gel columns (Pierce). Rabbit polyclonal anti-HMGB1 antibodies were from Pharmingen BD.

Proliferation assay

Cells were seeded in 6-well plates ($1x10^5$ cells/well) and grown in RPMI supplemented with 20% FCS. After 24 hours the medium was replaced with serum-free RPMI for 16 hours. Subsequently the cells were grown with medium alone, or medium with the addition of 20% FCS or HMGB1 at the concentration of 1, 3, 10, and 30 ng/ml. Cells were counted after 1, 2 and 3 days, and Trypan blue dye exclusion was used as indicator of cell viability. All experiments were performed three times in duplicate.

Chemotaxis assay

Cell migration was assayed using Boyden chambers. Briefly, PVP-free polycarbonate filters with 8 μm pores (Costar) were coated with 5 μg/ml porcine skin gelatin (Sigma). Serum-free RPMI (negative control), RPMI containing 10, 50 or 100 ng/ml HMGB1, and RPMI with 20% serum (positive control) were placed in the lower chambers. D16 cells were grown in RPMI plus 10% FCS, starved overnight, washed twice with PBS to eliminate any floating cells, and harvested with trypsin. Fifty thousand cells rsuspended in 200 μl RPMI were placed in the upper chambers and incubated at 37°C in 5% CO_2 for 16 h. Cells remaining on the upper surface of the filters were mechanically removed, those which had migrated to the lower surface were fixed with ethanol, stained with Giemsa Stain Modified (Sigma) and counted at 400x magnification in ten random fields per filter. Assays were performed in triplicate and repeated three times in independent experiments.

Transmigration assay

BAEC cells were grown in DMEM plus 10% FCS on polycarbonate transwell inserts (3 μm pores; Costar) for 5 days until they formed a

monolayer. The inserts were then placed between chambers in Boyden apparatuses, and the tightness of monolayers was checked by measuring the diffusion of BSA between chambers. Mesoangioblasts (10^5 cells in 100 µl RPMI) were placed in the upper compartments and RPMI containing various concentration of HMGB1 or VEGF was placed in the lower compartments (500 µl). After 16 hours at 37°C the filters were removed and stained with Giemsa Stain Modified. The number of the cells that had migrated through the monolayer was determined as indicated for the chemotaxis assays. These experiments were carried out three times in duplicate.

Immunoblot analysis

Western blots to quantify HMGB1 were performed as described by Degryse, B. et al. ["The high mobility group (HMG) boxes of the nuclear protein HMG1 induce chemotaxis and cytoskeleton reorganization in rat smooth muscle cells." *J. Cell Biol.* **152**, 1197-2006 (2001)], using rabbit polyclonal anti-HMGB1 antibodies from Pharmingen BD.

Flow cytometry

Mesoangioblasts starved overnight were grown in RPMI medium alone, RPMI plus 20% FCS or RPMI plus 100 ng/ml HMGB1 for 6, 12, 24 or 48 hours. Cells were washed, fixed in 70% ethanol, stained with 50 µg/ml propidium iodide (PI) in PBS plus 50 µg/ml RNase A and incubated for 30 min at room temperature. The DNA content was measured by flow cytometry (FACScan; Becton-Dickinson) using the Standard CellQuest software.

Estimation of the number of cell divisons using CFSE staining

Ten million embryonic or adult mesoangioblasts were seeded on 100 mm dishes in RPMI supplemented with 20% FCS. After 24 hours the cells were starved in RPMI alone overnight. Cells were then washed in PBS and CFSE was added to the final concentration of 2.5 M for 8 minutes at room temperature. The staining was quenched by the addition of 10% FCS and cells

were washed in RPMI. Fluorescently labeled cells were then grown in RPMI alone, RPMI plus 100 ng/ml HMGB1 or RPMI plus 20% FCS and harvested after 48 hours. Cells were then analyzed on FACScan.

Cytoskeleton visualization

BAEC cells were grown on glass coverslips until fully confluent. After the treatments described in the text, the cells were washed with PBS and fixed with 4% paraformaldehyde at room temperature for 10 minutes. Cells were then stained with FITC-conjugated phalloidin (Sigma) to visualize the actin cytoskeleton as described.

Preparation of HMGB1-loaded beads

Heparin beads (34 μm diameter) were recovered from a HiTrap Heparin HP column (Pharmacia) and extensively washed in PBS. Beads (20 μl packed volume) were then incubated for 1 hour at 4°C with 60 μg HMGB1, harvested by centrifugation, washed twice with PBS and resuspended in PBS. SDS-PAGE was performed to check the amount of HMGB1 on heparin beads.

Intra-artery delivery of mesoangioblasts in mice

Heparin beads (a slurry containing 3 μg beads in 20 μl PBS), either laoded with HMGB1 or not, were injected with an insulin syringe into *tibialis anterior* muscles of 6-week old female CD-1 mice (3 per group). After 1 hour mesoangioblasts (4x105 cells/animal) were injected through the femoral artery as previously described; animals were sacrified 24 hours later. For histochemistry analysis, samples of *tibialis anterior* muscles were frozen in liquid nitrogen-cooled isopentane and cryostat-sectioned. Serial muscle sections of 10 μm thickness were stained with X-gal (for the experiment with LacZ labeled cells), or directly visualized (for the experiment with DiI). DiI was from Molecular Probes, USA.

RESULTS

HMGB1 stimulates the proliferation of vessel-associated embryonic stem cells.

Stem cells isolated from mouse dorsal aorta of E9.5 embryos (mesoangioblasts) were cultured in vitro and tested for the presence of the CD34, Kit, Flk1 and MEF2D cellular markers. We used one of these clones (called D16) to assess whether HMGB1 can act as a mitogen.

D16 cells were seeded in RPMI medium with 20% FCS and then starved for 16 hours in the absence of serum to synchronize the cell population. Increasing concentrations of HMGB1 were then added to the medium without serum, and cells were counted after 1, 2, and 3 days. There is a significant increase in the number of D16 mesoangioblasts after stimulation with HMGB1 up to day 2, while only a weak proliferative effect is observed between days 2 and 3. All concentrations tested had similar effects. HMGB1-stimulated D16 cells had a normal morphology and excluded Trypan blue up to the end of the experiment, whereas cells in control cultures without HMGB1 were dying. A small percentage of D16 cells treated with HMGB1 were still dividing at day 3, whereas none of the negative control cells was. HMGB1 has no mitogenic effect on 3T3 fibroblasts.

We investigated in more detail the proliferative response of D16 cells to HMGB1: cells were exposed for 6, 12, 24 and 48 hours to RPMI medium alone (negative control), or medium containing HMGB1 or 20% FCS, and analyzed for DNA content by FACS after propidium iodide staining. After six hours of stimulation with HMGB1, the majority of mesoangioblasts had entered the cell cycle; after 48 hours, most cells appeared to have a diploid DNA content and thus to be in G1 or G0 (Fig. 1B). We evaluated the number of cell cycles triggered by HMGB1 by staining at time 0 the cell membranes with the fluorescent dye CFSE, and determining after 48 hours by FACS the

number of cells that had one-half of the initial quantity of dye (1 division) or one-quarter (two divisions). About half of the cells appeared to have divided once, and half twice. By comparison, all cells cultured in 20% FCS had divided at least twice.

These data indicate that HMGB1 induces a limited number of cell divisions. This might be due to a specific program of mesoangioblasts, or to the depletion of HMGB1 in the medium. We therefore tested whether continued addition of HMGB1 could sustain cell growth. HMGB1 was added at 30 ng/ml at time 0; cells stimulated once (HMGB1+) received no further addition of HMGB1, whereas cells stimulated continually (HMGB++) received additional HMGB1 at 12, 36 and 60 hours. Cells stimulated once divided once and then levelled off; re-exposed cells continued to divide. After 48 hours, no HMGB1 was detectable by Western blot in the medium of cells stimulated once, while HMGB1 equivalent to about 40 ng/ml remained in the medium of multiply-exposed cells. Taken together these results indicate that HMGB1 acts as a growth factor for D16 cells, but is rapidly depleted.

HMGB1 induces mesoangioblast migration

We have previously shown that HMGB1 is a chemoattractant for rat smooth muscle cells (RSMC). We then investigated whether HMGB1 is a chemoattractant for D16 cells too. In a chemotaxis assay using modified Boyden chambers, HMGB1 stimulated migration of D16 cells in a concentration-dependent manner. Polyclonal antibodies against HMGB1, but not nonspecific control antibodies, totally blocked the migratory response.

We also tested the individual domains of HMGB1 for the ability to induce D16 cell migration. Neither box A nor box B alone induced appreciable migration. The didomain fragment (comprising boxes A and B) had a non-significant effect, whereas the ABbt fragment, that only lacks the acidic tail, was as potent as the full-length protein. Huttunen et al. ["Receptor

for Advanced Glycation End Products-binding COOH-terminal Motif of Amphoterin Inhibits Invasive Migration and Metastasis." *Cancer Res* 62, 4805-4811. (2002)] have identified residues 150-183 as the HMGB1 segment that interacts with RAGE. Remarkably, anti-HMGB1 antibodies that specifically interact with the amino acid stretch between box B and the acidic tail abolish D16 cell migration, whereas antibodies that specifically recognize box A have no effect. Our data therefore indicate that HMGB1 is a powerful chemoattractant for D16 cells, and suggest that RAGE is its receptor. RNA profiling of D16 cells (not shown) indicate that they express RAGE, and RAGE protein is detectable in D16 cells by Western blot.

HMGB1 induces mesoangioblast migration across endothelial monolayers

Mesoangioblasts are vessel-associated stem cells that can migrate to damaged tissues through the general circulation, and have the ability to transit through the endothelial barrier. We then tested whether HMGB1 could also promote the transmigration of stem cells across an endothelial monolayer grown on the septum between the chambers of a Boyden apparatus. When we added 100 ng/ml HMGB1 to the medium in the lower chamber, the number of D16 cells crossing the monolayer increased 4-fold when compared to medium without any addition. HMGB1 has higher potency than VEGF, a signaling molecules is known to promote cell migration across endothelial barriers.

Several studies have shown that vascular endothelial growth factor (VEGF) induces profound cytoskeletal reorganization of endothelial cells characterized by the formation of transcytoplasmic stress fibers. Furthermore, treatment with VEGF destroys adherens junctions, which are important to maintain the endothelial barrier function. Thus, we compared the effect of HMGB1 on endothelial cells to that of VEGF. After stimulation with HMGB1 for 5 to 30 minutes, endothelial cells were fixed, labeled with fluorescein–

conjugated phalloidin and examined by immunofluorescence. Both HMGB1 and VEGF caused stress fiber formation and disaggregation of endothelial cells, suggesting that HMGB1 can dramatically increase the permeability of the endothelial lining of vessels.

HMGB1 directs mesoangioblast homing *in vivo*

Having established that HMGB1 is a chemoattractant for mesoangioblasts in vitro, we decided to assess its ability to control mesoangioblast migration *in vivo*. Heparin beads were loaded with HMGB1 at the concentration of 3 μg/ml and then injected with a fine needle into the *tibialis anterior* muscle of mice. D16 cells transduced by a lentiviral vector causing the expression of nuclear LacZ were injected after 30 min through the proximal femoral artery (see materials and methods). The mice were sacrificed after 24 hours and the *tibialis anterior* muscle was removed, sectioned, and analyzed by immunohistochemistry. Muscles injected with HMGB1-loaded beads showed a considerable swelling compared to both sham-injected muscles and muscles injected with unloaded heparin beads, suggesting that HMGB1 caused considerable muscle inflammation. This is consistent with HMGB1's role as proinflammatory cytokine.

Muscle sections were stained with X-gal and blue cells were scored using computer-assisted imaging techniques. Large groups of blue cells were found in the vicinity of HMGB1-loaded beads; a minority of sections displayed individual blue cells dispersed throughout the muscle. The sections from muscles injected with unloaded beads had no blue cells at all.

These observations clearly suggest that HMGB1 is able to recruit mesoangioblasts in vivo.

The biological action of HMGB1 on adult mesoangioblats

These findings identify a role for extracellular HMGB1, a proinflammatiory cytokine, on the migration and proliferation of embryonic

mesoangioblasts. However, inflammation does not occur in the embryo even in the presence of tissue damage. We then asked whether HMGB1 had an effect on adult mesoangioblasts as well.

We repeated the experiments described previously on adult mesoangioblasts isolated from bone marrow (G1 clone, see materials and methods). HMGB1 causes adult stem cell proliferation (Panel A), chemotaxis and transmigration (panel B). Finally, like embryonic mesoangioblasts, adult mesoangioblasts can be recruited by HMGB1 into the *tibialis anterior* muscle.

Similar effects were also observed in additional experiments with different adult mesoangioblast lines derived from the aorta of 2-week old mice.

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CLAIMS

1. A method for isolating and expanding multipotent stem cells (mesoangioblasts) which comprises the steps of:

a) providing vascular cells by dissociation of cultured embryonic, fetal, perinatal or adult mammalian vessels;

or

a') selecting ckit+ cells from postnatal or adult mammal bone marrow or cord blood;

or

a'') selecting AC133+/CD34+ cells from the outgrowth of vessels within fragments of skeletal muscle tissue maintained in culture without changing the medium for a period of at least one week;

b) cloning the cells obtained from a), a') or a'') at limiting dilution on a feeder layer of Mitomycin C-treated primary fibroblasts;

c) selecting and growing positive clones and, optionally,

d) repeating culture passages on the feeder cell layer and separating the expanded clones from fibroblasts.

2. A method according to claim 1, wherein the vascular cells are enzymatically dissociated from the explanted tissues.

3. A method according to claim 1, wherein embryonic fibroblasts are used as feeder cells.

4. A method according to claim 3, wherein STO fibroblasts are used.

5. A method according to claim 1, wherein the positive clones are selected by previous labeling the dissociated tissues with a dye allowing for single cell detection.

6. A method according to claim 5, wherein said dye is the fluorochrome DiI.

7. Isolated pluripotent mesoangioblast stem cells obtainable by the method of claims 1-6.

8. Mesoangioblast stem cells according to claim 7, characterized by the expression of the markers cKit, Flk-1, Sca1, MEF2D, GATA-4, CD34 and by the absence of expression of Myf5, Nkx2.5, Cbfa1, Oct-4, epithelial antigens, alkaline phosphatase.

9. Human mesoangioblast stem cells according to claims 7-8.

10. Mesoangioblast stem cells according to claims 7-9, containing a lentiviral vector encoding a biologically active protein.

11. A method for in vitro conversion of the mesoangioblast stem cells of claims 7-9 into a differentiated cell-type or tissue which comprises treating said stem cells with one of more growth factors, cytokines, hormones, vitamins.

12. A method for in vitro conversion of the mesoangioblast stem cells of claims 7-9 into a differentiated cell-type or tissue which comprises co-culturing said stem cells with cells of said differentiated cell-type or tissue.

13. A method according to claims 11-12, wherein said differentiated cell-type or tissue is selected from endothelium, osteoclasts, smooth, cardiac and skeletal muscle, cartilage and bone tissues.

14. A method according to claim 13, for the conversion of mesoangioblasts into muscle tissue in the presence of biosynthetic extracellular matrix.

15. The use of mesoangioblast stem cells according to claims 7-9 for the ex-vivo preparation of mesodermal tissues.

16. The use of mesoangioblast stem cells according to claims 7-9 for the preparation of a therapeutic agent for the treatment of pathologies that require tissue or cell repair or regeneration.

17. The use according to claim 16, wherein the therapeutic agent further comprises HMGB1.

18. The use according to claim 15, wherein said pathologies are muscular dystrophies, and myocardial infarction.

19. The use of mesoangioblast stem cells according to claims 7-9 for the preparation of pro-angiogenic agents.

20. The use of mesoangioblast stem cells for setting up in vitro assays for the identification of genes and proteins involved in the differentiation into cardiac or other mesodermal tissues or in the maintenance of stemness.

21. The use of claim 20, wherein said in vitro assays are carried out with microarrays or differential proteomics screenings proteomics assays or with antibodies libraries.

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FIGURE 1

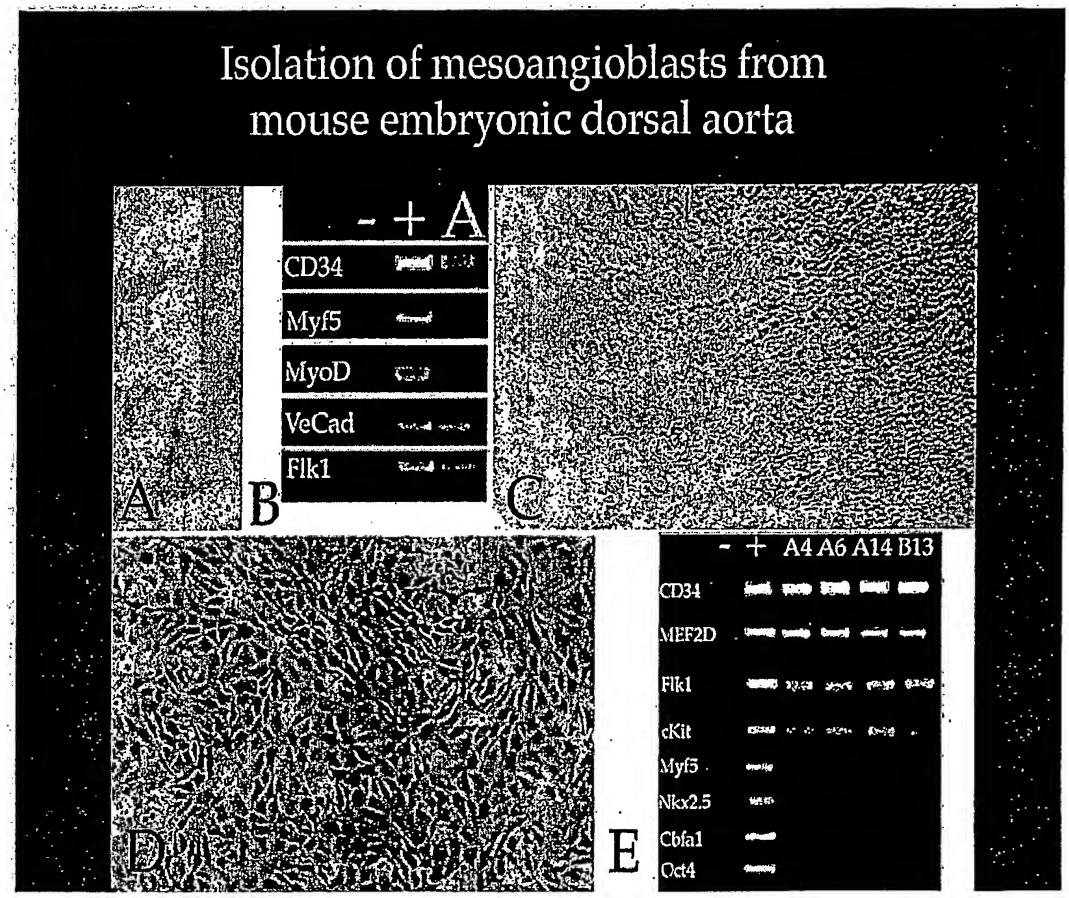

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FIGURE 2

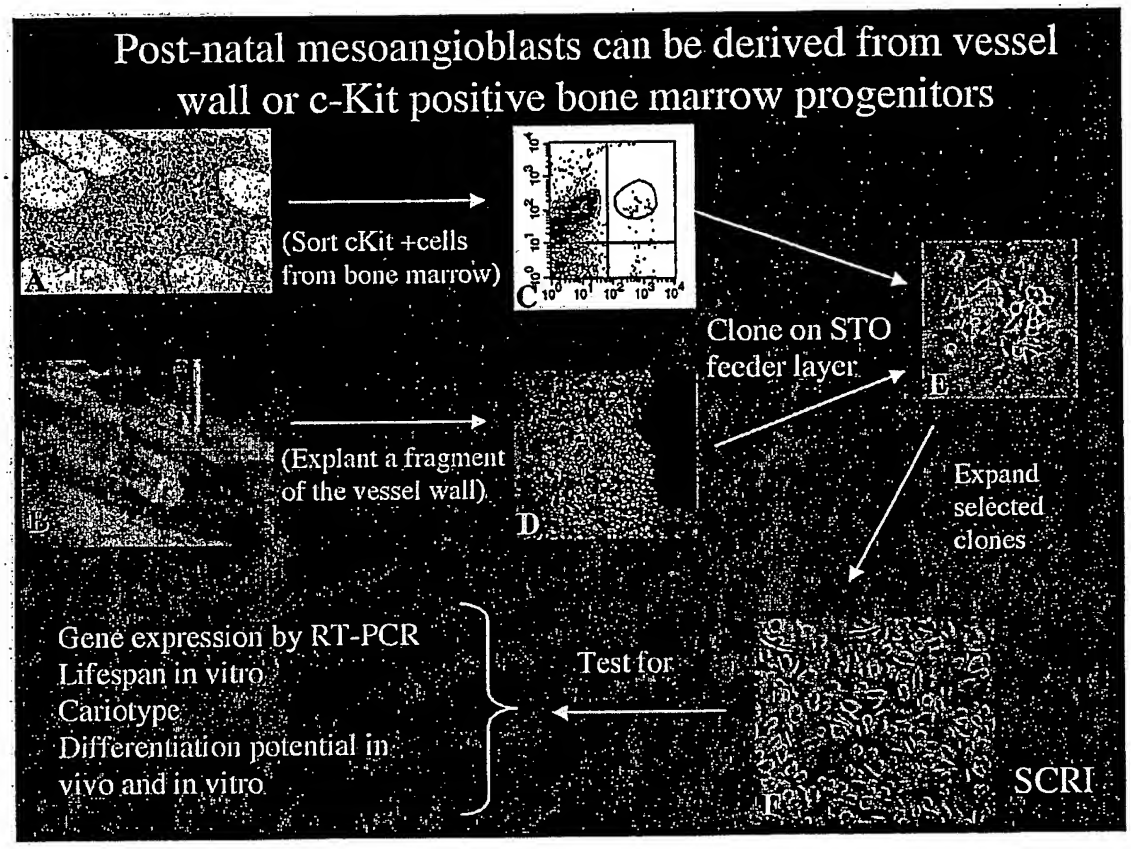

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FIGURE 3

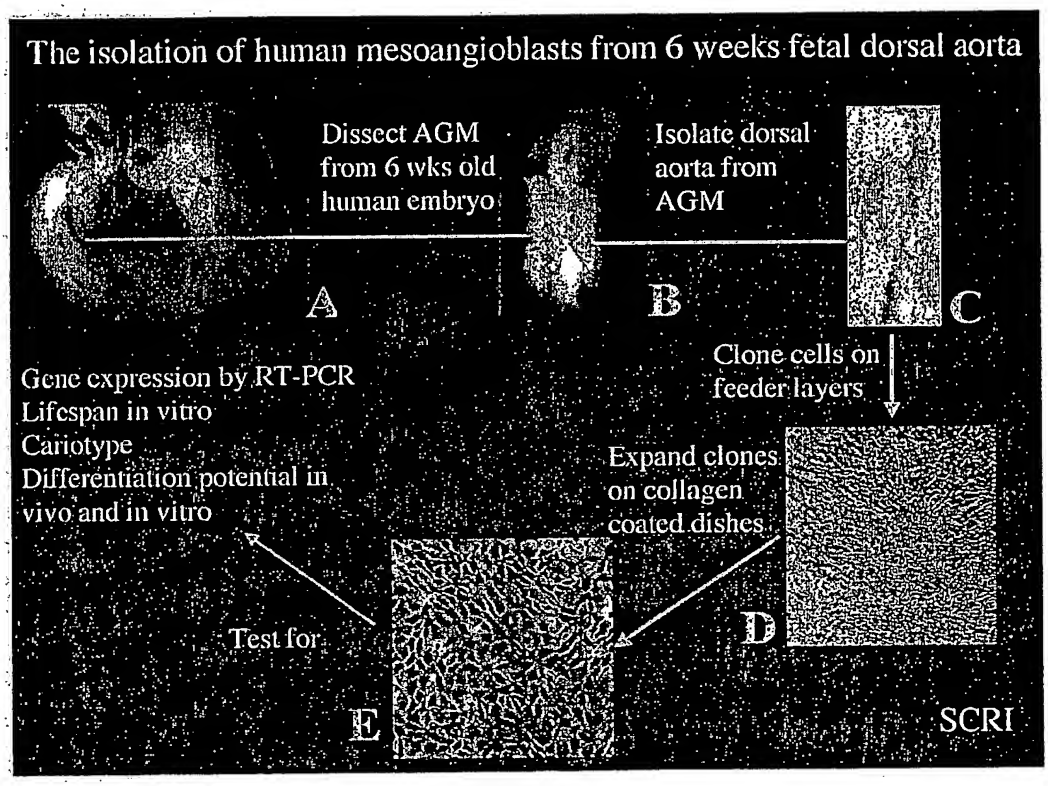

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FIGURE 4

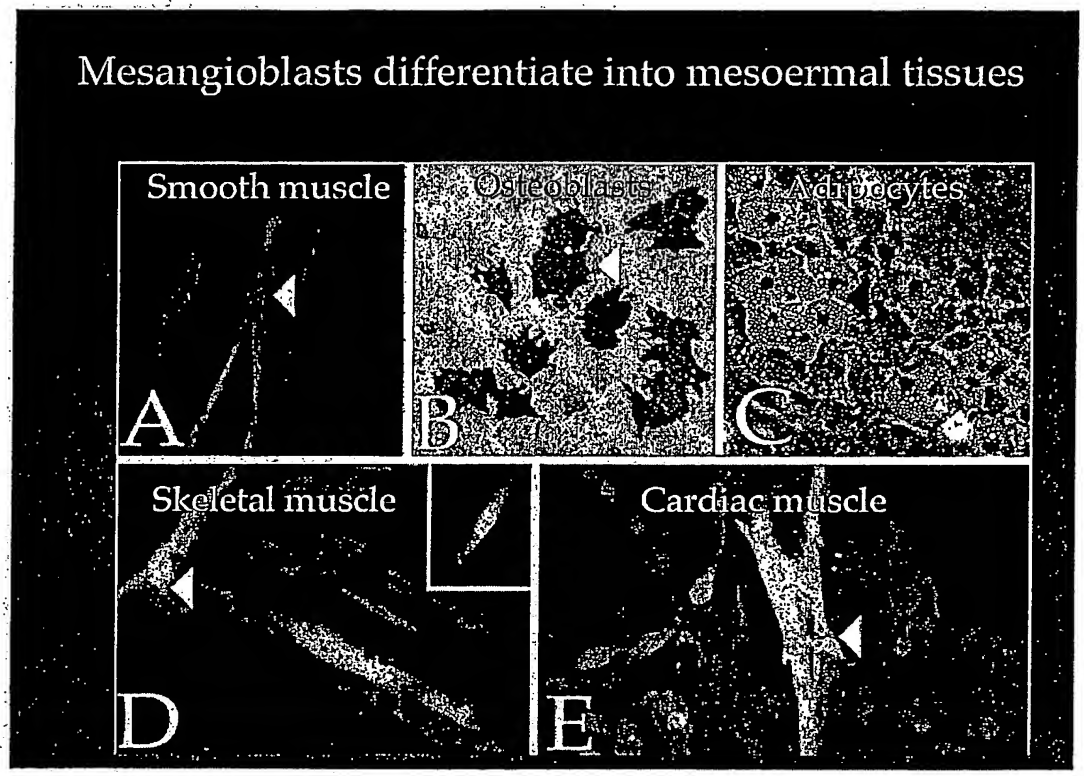

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FIGURE 5

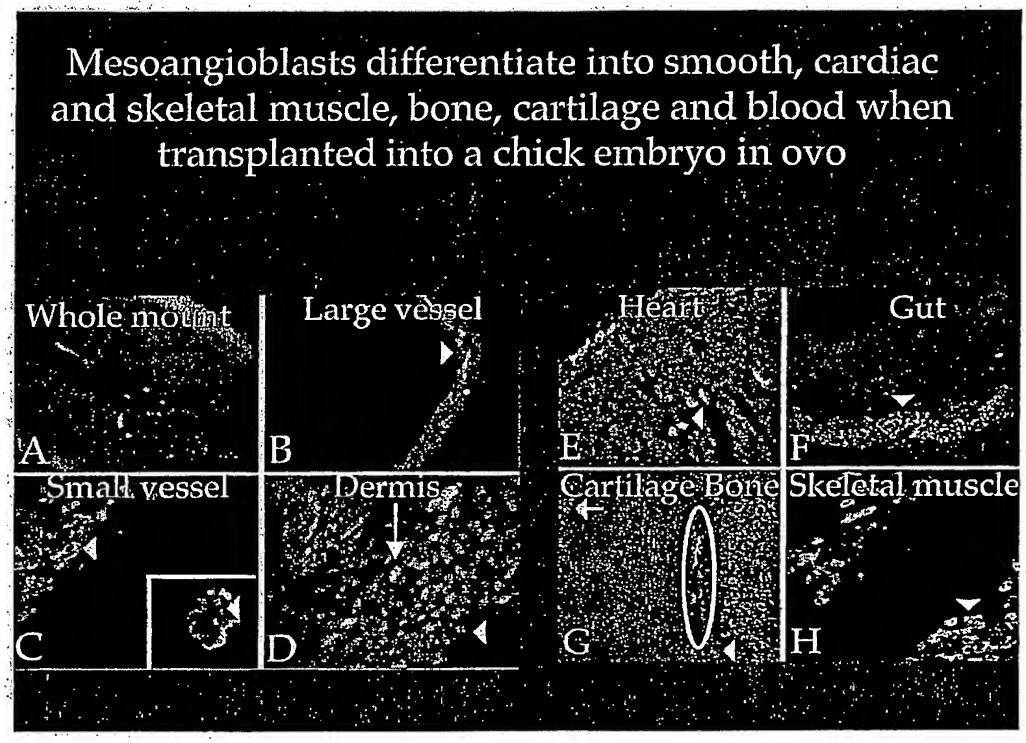

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FIGURE 6

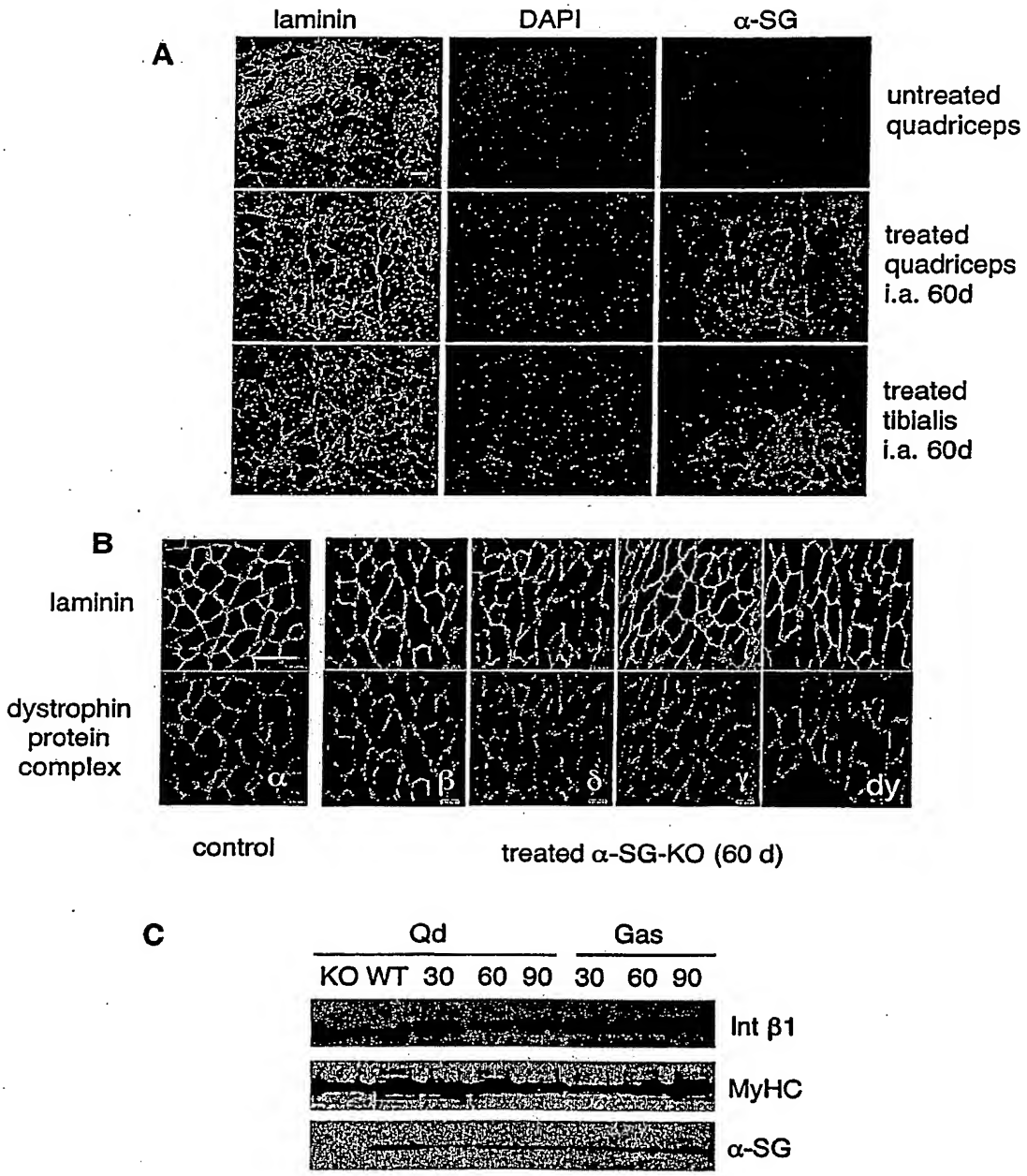

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FIGURE 7

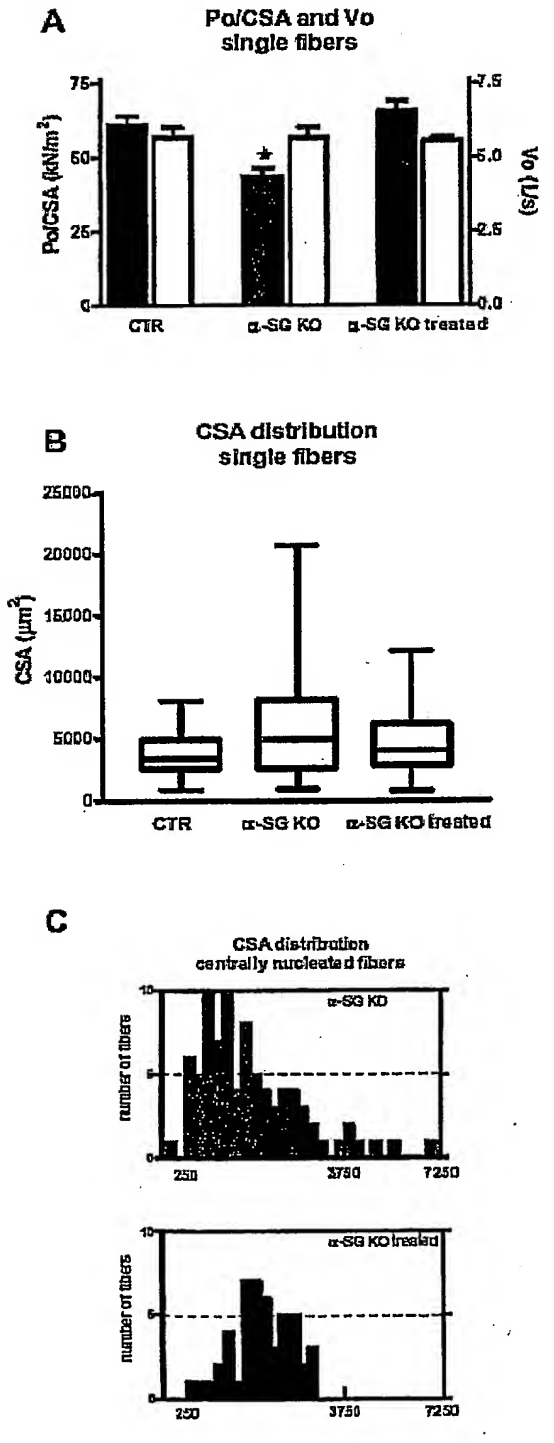

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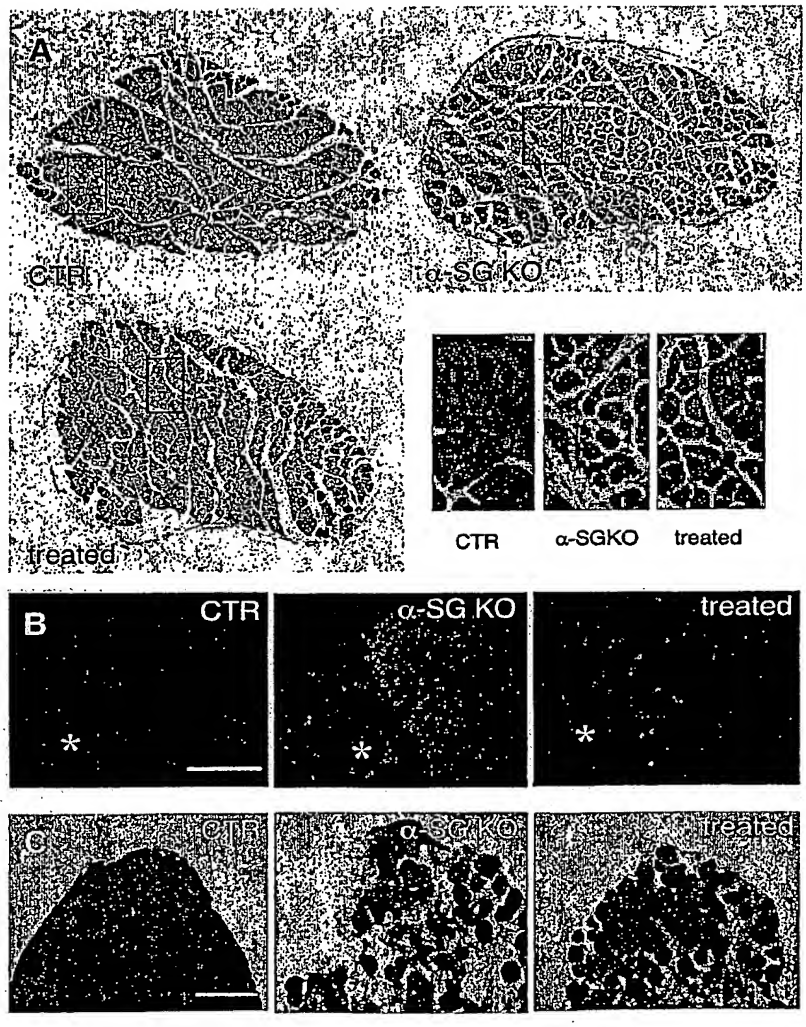
FIGURE 8



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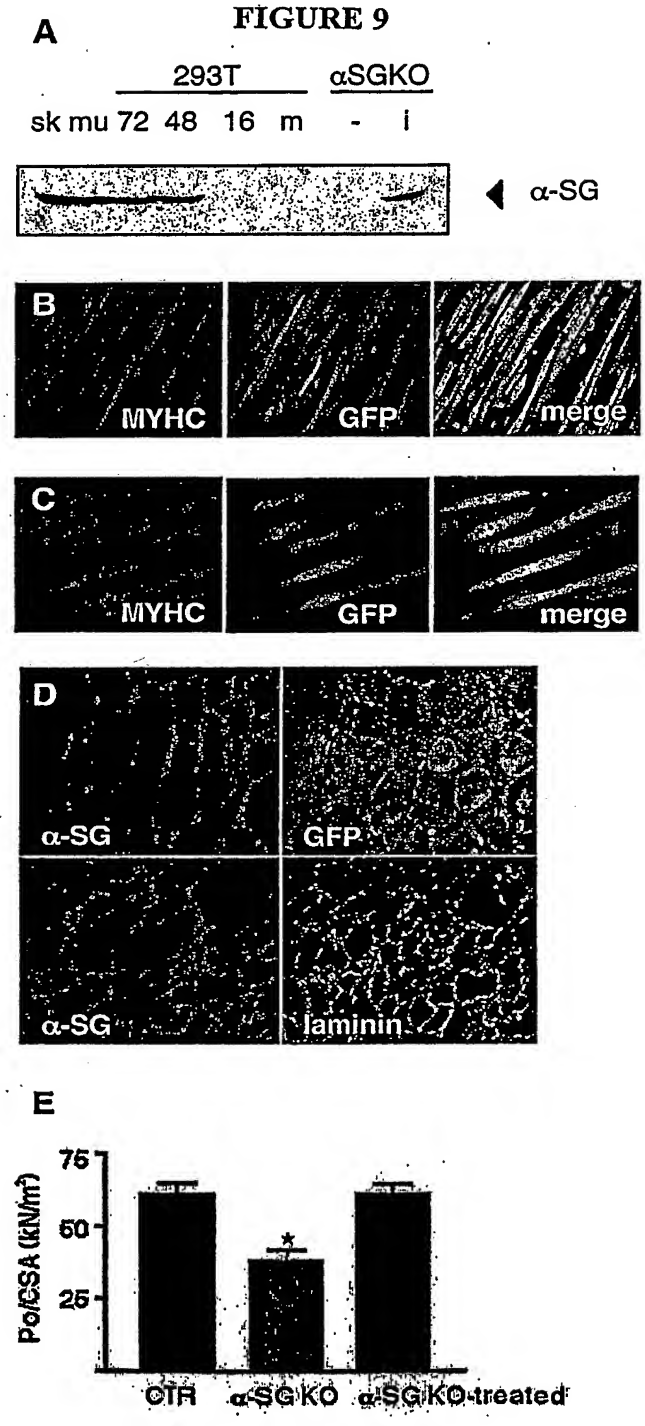
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D
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GFP
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75
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Po/CSA (kN/m²)
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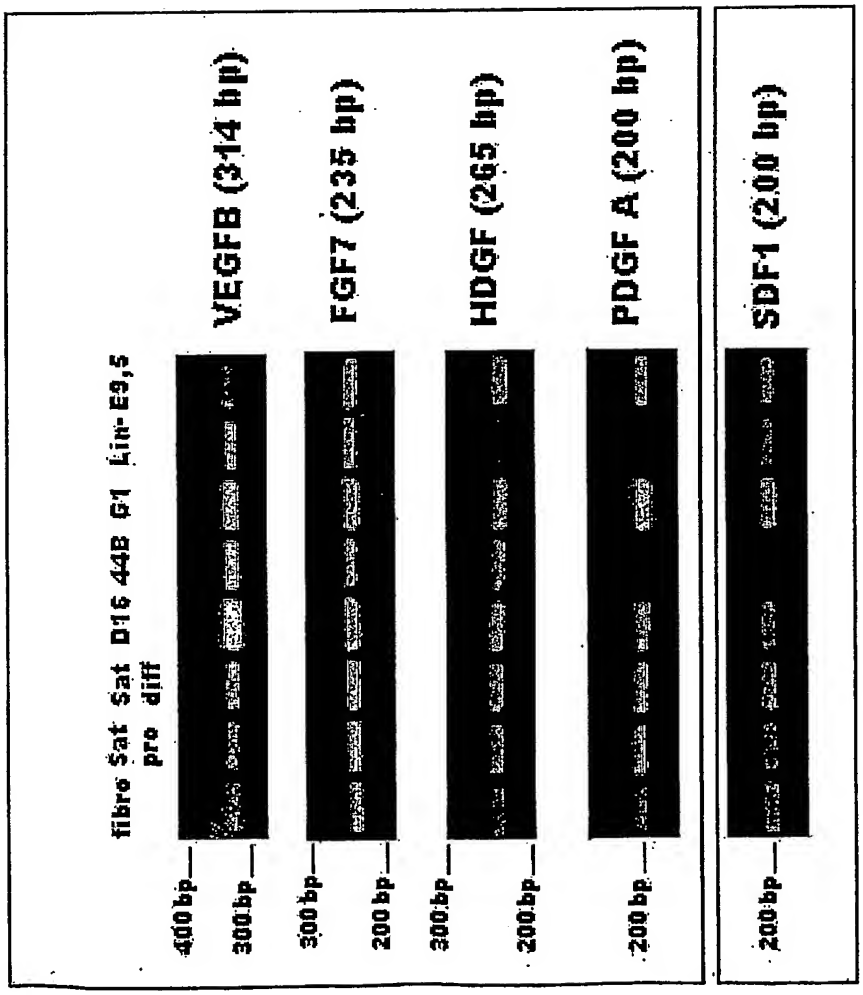
CTR
α-SG KO
α-SG KO-treated

FIGURE 10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INTERNATIONAL SEARCH REPORT

Interna Application No
PCT/EP 03/04976

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/06 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
BIOSIS, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	MINASI MARIA G ET AL: "The meso-angioblast: A multipotent, self-renewing cell that originates from the dorsal aorta and differentiates into most mesodermal tissues." DEVELOPMENT (CAMBRIDGE), vol. 129, no. 11, June 2002 (2002-06), pages 2773-2784, XP002250303 June, 2002 ISSN: 0950-1991 the whole document --- -/--	1-21

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Date of the actual completion of the international search	Date of mailing of the international search report
14 August 2003	08/09/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Friedrich, C

INTERNATIONAL SEARCH REPORT

Internat Application No
PCT/EP 03/04976

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	DE ANGELIS LUCIANA ET AL: "Skeletal myogenic progenitors originating from embryonic dorsal aorta coexpress endothelial and myogenic markers and contribute to postnatal muscle growth and regeneration." JOURNAL OF CELL BIOLOGY, vol. 147, no. 4, 15 November 1999 (1999-11-15), pages 869-877, XP002250337 ISSN: 0021-9525 the whole document	1-21
A	DZIERZAK E: "A dynamic system" LANCET, XX, XX, vol. 358, no. 1, 22 December 2001 (2001-12-22), page 31 XP004378693 ISSN: 0140-6736 the whole document	1-21

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